CHOLESTEROL TRANSPORT GENE

CROSS REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional patent applications Ser. No. 60/162,803 filed November 1, 1999 and Ser. No. 60/215,564 filed June 20, 2000.

BACKGROUND OF THE INVENTION

Cholesterol is one of the most intensely studied of molecules that circulate in the human bloodstream. Cholesterol is a lipid that is a major component of cell membranes and also is the precursor of steroid hormones and the bile acids. Two sources of cholesterol are available to cells. Endogenous cholesterol is synthesized in the liver and other cells and transported through the bloodstream to other cells. Since cholesterol is highly apolar, it is transported through the bloodstream in the form of lipoproteins consisting essentially of a core of apolar molecules such as cholesterol surrounded by an envelope of polar lipids, primarily phospholipids. Alternatively, exogenous cholesterol may be absorbed from the gut. Exogenous cholesterol is transported from the lumen of the gut into the blood or lymph for distribution via lipoprotein particles to other cells of the body.

For the diagnostic purposes related to human health, the lipoproteins are classified into several categories based on the density of the lipoprotein particles. The two categories most discussed in connection with human health are the low-density lipoproteins (LDL) and the high-density lipoproteins (HDL). For many people, HDL is known as the "good cholesterol" since it has a somewhat protective effect on the tendency of LDL to contribute toward coronary artery disease and related cardiovascular conditions such as stroke. Studies have shown an inverse relationship between levels of serum HDL and the occurrence of coronary artery disease, resulting in HDL levels being graded as a strong risk factor for cardiovascular disease prediction. Accordingly, a low level of HDL cholesterol, referred to as hypoalphalipoproteinemia, is a blood abnormality that correlates with increased risk of cardiovascular disease.

One rare form of genetic HDL deficiency is known as Tangier disease. Patients with the homozygous form of this disease have an almost total absence of serum HDL cholesterol. The disease is an autosomal recessive trait, and patients with the disease accumulate cholesterol esters in several tissues, resulting in characteristic physical features

including enlarged orange or yellow tonsils, hepatosplenomegaly, peripheral neuropathy, and cholesterol deposition in the rectal mucosa. The symptoms of the disease appear to be attributable to a deficiency in cholesterol and/or phospholipid transport across cell membranes, principally out of cells that manufacture or store excess cholesterol. The orange tonsils are, for example, caused by the accumulation of cholesterol esters and related carotenoids in macrophages. It has now been established that Tangier Disease is a monogenic disorder caused by a mutation in the ABC1 gene (Brooks-Wilson, A. et al. 1999, "Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency." *Nat. Genet.* 22:336-345; Bodzioch, M..et al. 1999, "The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier Disease." *Nat. Genet.* 22:347-351; Rust, S., et al. 1999, "Tangier Disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1." *Nat. Genet.* 22:352-355). Other patients exhibit a more common form of genetic HDL deficiency which results in low plasma HDL and premature cardiovascular disease, but an absence of the severe symptoms associated with Tangier disease. A large sub-group of patients with low HDL have an inherited form of this disease, familial hypoalphalipoproteinemia (FHA). It has been found that many of these patients are heterozygotes for mutations in ABC1. (Brooks-Wilson, A. et al. 1999, supra). Thus, ABC1 in its homozygous form causes Tangier disease and in its heterozygous form causes FHA.

An animal model for low HDL conditions exists in the form of the Wisconsin Hypo-Alpha Mutant (WHAM) chicken. This single gene mutation arose naturally and was identified because of the white skin phenotype and a closed flock of the chickens has been maintained as an animal model for low HDL disease. (Poernama et al. *Jour. Lipid Res.* 31:955-963 (1990)). The effect of this mutation on diet-induced atherosclerosis has been investigated, and it has been found that WHAM chickens are highly deficient in their ability to transport cholesterol from the gut into the blood. (Poernama et al. *Arteriosclerosis and Thrombosis* 12:2:601-607 (1992)). Some efforts have been made to identify the genetic element responsible for the mutation in the WHAM chickens (Schreyer et al. *Arteriosclerosis and Thrombosis* 14:12:2053-2059 (1994)), but prior to the instant invention, these efforts have not been successful.

It is highly desirable to identify and develop compounds and therapeutic agents which are useful for reducing cholesterol transport from the gut to the blood or lymph and for the regulation and treatment of cardiovascular disorders (such as high LDL or serum cholesterol levels), obesity, elevated body-weight index and other disorders relating to lipid metabolism.

SUMMARY OF THE INVENTION

The present invention is summarized in that a method is described for the lowering of levels of LDL cholesterol in an individual comprising administering to the individual an agent which modulates the activity of the ABC1 protein in the intestinal cells of the individual.

The present invention is further summarized in that a method is described for reducing cholesterol transport from the gut into the blood or lymph comprising administering a modulator of the ABC1 protein. In a preferred embodiment, the modulator is an inhibitor of ABC1 activity, and it is administered orally.

The present invention is also summarized in that a method for screening drug candidates for lowering serum LDL levels or for reducing cholesterol transport from the gut into the blood or lymph includes the steps of screening compounds for the effect of modulating ABC1 protein activity. In a preferred embodiment, the modulator is an inhibitor of ABC1 activity. In a further embodiment, successful candidates are further screened for the effect of not stimulating insulin production. Successful drug candidates may optionally be further modified by combinatorial chemistry to generate preferred therapeutic agents.

Compositions of the invention include compounds which are useful for reducing cholesterol transport from the gut to the blood or lymph and for the regulation and treatment of cardiovascular disorders (such as high LDL or serum cholesterol levels), obesity, elevated body-weight index and other disorders relating to lipid metabolism which are identified using the screening assays of the invention.

Other objects, advantages and features of the present invention will become apparent from the following specification.

BRIEF DESCRIPTION OF THE DRAWING FIGURES

Fig. 1 is a graphical illustration of the relationship between the human ABC1 gene and the mutation responsible for the phenotype of the WHAM chickens.

Fig. 2 is a graphical representation of some of the data from an example below.

Fig. 3 is an electro-micrograph of the intestinal wall demonstrating accumulation of giant lipid droplets in WHAM chickens but not control chickens. The lipids are not found in columnar epithelial cells, but in deeper underlying cells of the lamina propria.

Fig. 4 is a graphical representation of data which demonstrates the response to dietary cholesterol in normal and WHAM chickens

DETAILED DESCRIPTION OF THE INVENTION

The insights that gave rise to the present invention derive from several sources. One is the recent identification by several of the inventors of the specific human gene responsible for Tangier disease. That gene was identified to be a gene known as ABC1, a gene that is part of the ATP-binding cassette superfamily of genes. Deficiencies in the function of this gene are associated with decreased transport of cholesterol out of cells that synthesize excess cholesterol. The second insight was the understanding that the genetic mutation in the WHAM chickens is, in fact, a mutation in the same ABC1 gene or a related gene. This insight establishes for the first time that the ABC1 protein may be critical for cholesterol absorption. Because the mechanism of action of the mutant gene in the WHAM chickens had been previously understood to be a deficiency in absorption of isoprenoids and sterols from the lumen of the digestive tract, this observation suggested to the inventors that the same ABC1 gene may be necessary for cholesterol transport from the digestive tract to the blood stream. The combination of these two observations had led to the understanding, first expressed here, that lowered serum cholesterol levels, and in particular, lower levels of LDL, of "bad" cholesterol, can be achieved by blocking the transport action of the ABC1 in facilitating the transport of cholesterol from the intestinal lumen through the intestinal wall cells into the blood stream. Since it is revealed here that the ABC1 gene is a necessary component in the transport of isoprenoids and sterols from the gut into the blood stream, as revealed in particular by the inability of WHAM chickens to uptake these compounds from their diets, it also becomes clear that uptake of these compounds in healthy individuals from the intestines can also be inhibited by blocking the action of otherwise functional copies of this transport protein. Since cholesterol in the intestinal tract is either secreted by the liver into the bile and thus into the intestinal tract, or derives from exogenous sources, blocking the absorption of cholesterol from the gut into the blood stream through the intestinal wall will result in lowering the total level of cholesterol in the plasma of the individual.

The ABC1 protein is a cross-membrane transport protein. In cells throughout the body, the protein transports cholesterol from the cytosol into the blood stream. In the cells of the intestinal wall, this transport protein performs a similar function. Cholesterol is absorbed from the intestinal lumen into the cells lining the intestinal wall and is then transported into the blood stream. In the absence of an effective ABC1 protein, the cholesterol can not be transported into the blood or lymph and can go no further. Thus, in ABC1 deficient individuals, cholesterol accumulates in the wall of the intestines. In individuals with normal ABC1 protein function, the ABC1 protein serves to transport the

cholesterol absorbed from the intestine from the cell into the blood stream. Thus inhibiting the transport function of the ABC1 protein in intestinal wall cells prevents cholesterol entering the gut from being transported into the blood stream.

This invention establishes for the first time the presence of a two stage process for cholesterol absorption from the gut: cholesterol first proceeds to cross the epithelial cells; and secondly by an ABC1-dependent process cholesterol is transported through the lamina propria into the blood or lymph. Thus an important aspect of this invention is the identification of a layer of cells beneath the columnar epithelium which are an essential part of the cholesterol absorption/transport pathway. These cells can be studied for other mechanisms or sites of activity for compounds which lead to inhibition of cholesterol absorption/transport from the gut to the blood.

This insight leads to other useful processes. Since the same gene, ABC1, is responsible for transport of cholesterol, phospholipids and other isoprenoids, including carotenoids, from intestinal wall cells into the blood stream, assays for the ability of a patient to transport of carotenoids from the gut into the serum will also be diagnostic of that person's ability to similarly transport cholesterol. Since mutations in ABC1 are a major cause of FHA, the carotenoid absorption test might constitute a clinically useful diagnostic procedure for identifying patients with ABC1 mutations and thus categorize patients for subsequent therapy.

The identification of a mutation in the ABC1 gene as the cause for Tangier disease was first reported only recently. See Brooks-Wilson et al. *Nature Genetics* 22:336-345 (1999), Bodzioch et al. *Nature Genetics* 22:347- 351 (1999), and Rust et al. *Nature Genetics* 22:352-355, (1999), each of which is hereby incorporated by reference. The ABC1 protein is a complex membrane protein with twelve transmembrane domains, that is a part of the ABC gene family, whose members include proteins implicated in the active transport of substances across biological membranes. The papers cited in this paragraph established the role of the ABC1 transporter gene as a necessary agent for the transport of cholesterol out across the membrane of a cholesterol producing cell. This document is the first report of the observation that the ABC1 gene is also necessary for the transport of cholesterol from the intestines to the blood stream and to teach a method to make use of that knowledge for human health purposes.

The establishment of the role for the ABC1 transporter protein in cholesterol uptake was made on the basis of the WHAM chickens, following the identification of the ABC1 gene as the causative agent for Tangier disease. It had been previously known that the mutant gene responsible for the mutant phenotype in the chickens mapped to the Z sex

chromosome, in the Y locus and proximal to the ID locus. Examination of the public mapping data from the chicken genome mapping project showed a region of synteny with a region of human chromosome 9 in which the human ABC1 gene is present. In short, genetic mapping has demonstrated that genes from syntenic loci are responsible for the mutation in the WHAM chickens and in the Tangier patients. DNA sequence anlysis has identified the mutation in the chicken ABC1 gene causitive of HDL deficiency. In the WHAM chickens, however, the symptoms appear to also be the result of deficiencies in uptake of substances (i.e. cholesterol and carotenoids) from the digestive tract. In the WHAM chicken, cholesterol ester accumulates in the wall of the intestine, but not in the columnar epithelium, rather in the lamina propria. ABC1 mRNA is present in this region in control chickens but is not seen in the columnar epithelium. This establishes the utility of the WHAM chickens as an animal model for the study of HDL and cholesterol transport deficiency in humans, and also provides the basis for the therapeutic and diagnostic strategies described in this document.

The WHAM chicken has thus supplied the first genetic evidence that vertebrates, like invertebrates, have an extracellular lipoprotein assembly pathway. Since the WHAM chickens are refractive to the effects of a high-cholesterol diet (see Examples, below), the inventors concluded that ABC1 plays a role in intestinal cholesterol transport.

The first and potentially most important strategy described here is based on the fact that if ABC1 is necessary for the transport of cholesterol from the intestines into the blood stream. Blocking the action of the ABC1 gene or protein in the cells of the intestinal wall from performing that transport activity results in decreasing the transport of cholesterol into the serum. Cholesterol normally enters the intestinal lumen from two sources, food eaten by the individual and from cholesterol excreted from the liver into the bile. If cholesterol transport is inhibited in the intestinal wall cells by an ABC1 blocker, serum cholesterol levels will go down, since the cholesterol secreted by the liver will not be re-directed into the blood stream. On the other hand, if the inhibition of cholesterol uptake is selectively performed only in the cells of the intestinal wall, there should be no effect on the levels of HDL in the individual's serum, since the normal transport of cholesterol out of cholesterol producing cells will not be affected. Since the site of ABC1 activity that is to be blocked is in the cells of the intestinal wall, and blockage of ABC1 activity elsewhere may not be desirable, it is envisioned that the most convenient mode of delivery of the ABC1 blocker will be by oral delivery.

It is envisioned that the transport activity of ABC1 can be inhibited in many ways. One method would be to inhibit the expression of endogenous ABC1 gene activity to

reduce the abundance of the ABC1 protein. An example of the implementation of this method would be an antisense construct for the ABC1 gene delivered (either in free form or by liposome or viral vector) through the intestinal tract to the intestinal wall cells. Another method would be to inhibit the activity of the protein by introducing a chemical inhibitor of the activity of the protein. An example of the second method would be the use of a drug containing a sulfonylurea compound, an agent known to inhibit ABC1 protein activity. In either case, the delivery methodology should be on capable of delivering the inhibiting agent to the cells of the intestinal lining.

For the modulation of the activity of ABC1 using genetic techniques, it is necessary to introduce the genetic elements into the cells of the intestinal epithelium. This can be done by using liposomes of viral vectors carrying the genetic elements orally. Such liposomes or viral vectors can achieve transfection of foreign genetic constructs into the somatic cells with which they come in contact as some frequency dependent on the efficiency of the particular vector. There are several methods that can be used to inhibit gene activity, but amongst those the best known is based on the used of an antisense RNA construct. A genetic construct can be made which encodes the coding region of at least a portion of the coding region of the native ABC1 gene, in the antisense direction. When such a construct is expressed in cells, the antisense RNA produced interferes with normal gene expression activity in the cells and the native levels of the targeted protein drop. Such an antisense technique can be used to selectively target unwanted cholesterol transport activity in the intestinal lining without interfering with desired ABC1 activity throughout the rest of the body. The sequence of the human ABC1 gene is appended hereto as SEQ ID:NO: 1 to enable the implementation of this strategy.

The sulfonylurea drugs act to inhibit ABC1. For example, one member of this drug family, glibenclamide, has been shown to inhibit iodide transport in frog oocytes which are induced to express ABC1. Becq et al. *Jour. Biol. Chem.* 272:5:2695-2699 (1997). Sulfonylurea drugs are also currently used in the treatment of diabetes to stimulate insulin secretion from islet cells in the pancreas. It is preferred that the sulfonylurea drug be one that is highly inhibitory of ABC1 activity but not one that stimulates insulin production. In this way, the drug could interfere with cholesterol uptake without unnecessarily stimulating insulin production. It is specifically envisioned that the family of sulfonylurea compounds can be screened to identify those members of the group which retain the ability to inhibit the activity of ABC1 without stimulating the production of insulin. The outline of a methodology for that screening process is described below.

Another use for the observation that the mutation in the ABC1 gene in the cause for

the phenotype in the WHAM chickens arises from the observation that the WHAM chickens were originally identified primarily because they cannot extract carotenoids from their gut, leaving the animals deficient in carotene (as a result, their serum is colorless instead of yellow and their skin is white instead of yellow).

Also, with the insight into the intestinal transport function of the ABC1 gene disclosed here, it becomes possible to screen new drugs for cholesterol lowering function. Chemical entities that will bind with high affinity to the extra-cellular domains of the ABC1 protein, such as domains identified above, will prove to have cholesterol lowering properties as long as they are capable of passing through the stomach into the intestines without deactivation or digestion. It is then possible to use the WHAM chickens as a control to test drugs identified in this fashion, since such drug should be ineffective in these chickens.

Another specifically envisioned class includes of inhibitors of ABC1 is antibodies, polyclonal or monoclonal, which are directed against the appropriate domains of the ABC1 transporter protein which are located on the surface of the intestinal cells. For the approach of using antibodies, it is preferred that the antibodies be raised against the domains of the ABC1 protein which appear to be exposed on the surfaces of those cells. Set forth in the sequence listing at the end of this document is the complete DNA sequence for the ABC1 gene and the amino acid sequence for the ABC1 transporter protein. The domains of the transported protein that are located exposed on the surface of the epithelial cells in the intestinal wall can be predicted based on computer analysis of this sequence information. The putative external domains of the ABC1 transporter protein identified by this means are set forth in the list following this paragraph. It is predicted that these regions are essential for the transport function of the ABC1 protein and that a protein or small molecule which binds to one of these regions (or to any other essential region of the ABC1 protein) will inhibit the transport activity of ABC1. To make antibodies against these regions, peptides can be prepared that include the amino acids sequences of these regions. These peptides can be used to make polyclonal antibodies by immunizing animals and recovering their serum. Monoclonal antibodies can be made as well. It is also envisaged that antibodies can be made by injecting the peptides into chickens and thus these chickens will produce eggs enriched in the needed antibody as in Yokoyama et al. *Am. J. Vet. Res.* 54:6:876-872 (1993). The antibodies can be recovered from the egg yolks and prepared separately, or the eggs themselves can be eaten by a patient, to expose the antibody to the target, i.e. the exposed domains of the ABC1 transporter protein. It is specifically envisaged that the resulting antibodies may be ingested by the individual being treated for introduction to the

target site. It has been previously demonstrated that antibodies may be introduced into an individual's food source to have selected effects on intestinal receptors, as is demonstrated by U.S. Patents Nos. 5,814,361 and 5,725,873, the specifications of which are hereby incorporated by reference. These patents disclose suitable methods for the delivery of antibodies in the diet to individuals to block an intestinal hormone.

Predicted external domains of ABC1

TM1-TM2	663 KEARLKETMRIMGLDNSI 680
TM3-TM4	740 FSRAN 744
TM5-TM6	795 ALFEEQGIGVQWDNLFESPVEEDGFN 820
TM7-TM8	1371
	FGKYPSLELQPWMYNEQYTFVSNDAPEDTGTLELLNALTKDP
	GFGTRCMEGNPIPDTPCQAGEEEWTTAPVPQTIMDLFQNGNW
	TMQNPSPACQCSSDKIKKMLPVCPPGAGGLPPPQRKQNTADI
	LQDLTGRNISDYLVKTYVQIIAKSLKNKIWVNEFRYGGFSLGV
	SNTQALPPSQEVNDAIKQMKKHLKLAKDSSADRFLNSLGRFM
	TGLDTRNNVKVWFNNKGWHAISSFLNVINNAILRANLQKGE
	NPSHYGITAFNHPLNLTKQQLSEVALMTTSVD 1654
TM9-TM10	1741 LLLLYGWSITPLMYPASFVFKIP 1763
TH11-TH12	1823 VKNQAMADALERFGENRFVSPLSWDLVGR 1851

ABC1 Nomenclature and Reported Nucleic Acid/Protein Sequences

The ABC1 gene and protein referred to herein is also sometimes referred to as ABCA1 or CERP (cholesterol-efflux regulatory protein) in the scientific literature. The complete ABCA1 cDNA, genomic DNA sequence, and predicted protein sequence has been disclosed in PCT/IBOO/00532 and US patent application Ser No. 09/654,328, filed September 1, 2000, incorporated herein by reference. The human ABCA1 in the GeneBank has the following accession numbers: AJ012376; AF165281; NM_005502; AF285167. Corresponding ABCA1 genes and peptides from other organisms have also been reported in GenBank.

Screening Assays for Modulators of ABC1 Activity

The invention provides screening assay methods for identifying therapeutic compounds useful for treatments which reduce exogenous cholesterol transport from the gut lumen to the blood or lymph and for the regulation and treatment of cardiovascular disorders (such as high LDL or serum cholesterol levels), obesity, elevated body-weight

index and other disorders relating to lipid metabolism which can be used in human patients. The screening assay methods of the invention simplify the evaluation, identification and development of candidate compounds and therapeutic agents for the treatment of such conditions and disorders. In general, the screening methods provide a simplified means for selecting natural product extracts or compounds of interest from a large population, generally a compound library, which are further evaluated and condensed to a few active and selective materials useful for treatments of such conditions and disorders (these treatments are sometimes referred to herein as the "desired purposes of the invention"). Constituents of this pool are then purified, evaluated, or modified by combinatorial chemistry in order to identify preferred compounds for the desired purposes of the invention.

Compounds that modulate ABC1 biological activity can be identified by their effects on a known biological activity of ABC1, including but not limited to cellular or microsomal scale assays of efflux of phospholipid, cholesterol or other chemical species, protein level assays of binding specificity, protein stability, regulated catabolism, or its ability to bind proteins, lipids or other factors, expression level or stability of ABC1 mRNA and precursor RNAs, or, in short, by any activity that identifies a biological effect, characteristic or feature of the ABC1 protein.

What follows is a general description of potential ABC1 screening assay. More detailed descriptions of certain of these assays are set out in a separate section below.

In one example, the phosphorylation state or other post-translational modification is monitored as a measure of ABC1 biological activity. ABC1 has ATP binding sites, and thus assays may wholly or in part test the ability of ABC1 to bind ATP or to exhibit ATPase activity. Drug screening assays could be based upon assaying for the ability of the protein to form a channel, or upon the ability to transport cholesterol or another molecule, or based upon the ability of other proteins bound by or regulated by ABC1 to form a channel. In addition to its role as a regulator of cholesterol levels, ABC1 may also transports anions. Functional assays could be based upon this property, and could employ drug screening technology such as (but not limited to) the ability of various dyes to change color in response to changes in specific ion concentrations in such assays can be performed in vesicles such as liposomes, or adapted to use whole cells.

Drug screening assays can also be based upon the ability of ABC1 or other ABC transporters to interact with other proteins. Such interacting proteins can be identified by a variety of methods known in the art, including, for example, radioimmunoprecipitation, co-immunoprecipitation, co-purification, and yeast two-hybrid screening. Such interactions

can be further assayed by means including but not limited to fluorescence polarization or scintillation proximity methods. Drug screens can also be based upon functions of the ABC1 protein deduced upon X-ray crystallography of the protein and comparison of its 3-D structure to that of proteins with known functions. Such a crystal structure has been determined for the prokaryotic ABC family member HisP, histidine permease. Drug screens can be based upon a function or feature apparent upon creation of a transgenic or knockout mouse, or upon overexpression of the protein or protein fragment in mammalian cells *in vitro*. Moreover, expression of mammalian (e.g., human) ABC1 in yeast or *C. elegans* allows for screening of candidate compounds in wild-type and mutant backgrounds, as well as screens for mutations that enhance or suppress an ABC1-dependent phenotype. Modifier screens can also be performed in ABC1 transgenic or knock-out mice.

Additionally, drug screening assays can also be based upon ABC1 functions deduced upon antisense interference with the gene function. Intracellular localization of ABC1, or effects which occur upon a change in intracellular localization of the protein, can also be used as an assay for drug screening. Immunocytochemical methods will be used to determine the exact location of the ABC1 protein.

Human and rodent ABC1 protein can be used as an antigen to raise antibodies, including monoclonal antibodies. Such antibodies will be useful for a wide variety of purposes, including but not limited to functional studies and the development of drug screening assays and diagnostics. Monitoring the influence of agents (e.g., drugs, compounds) on the expression or biological activity of ABC1 can be applied not only in basic drug screening, but also in clinical trials. For example, the effectiveness of an agent determined by a screening assay as described herein to decrease ABC1 gene expression, protein levels, or biological activity can be monitored in clinical trails of subjects exhibiting altered ABC1 gene expression, protein levels, or biological activity. Alternatively, the effectiveness of an agent determined by a screening assay to modulate ABC1 gene expression, protein levels, or biological activity can be monitored in clinical trails of subjects exhibiting decreased altered gene expression, protein levels, or biological activity. In such clinical trials, the expression or activity of ABC1 and, preferably, other genes that have been implicated in, for example, cardiovascular disease can be used to ascertain the effectiveness of a particular drug.

For example, and not by way of limitation, genes, including ABC1, that are modulated in cells by treatment with an agent (e.g., compound, drug or small molecule) that modulates ABC1 biological activity (e.g., identified in a screening assay as described herein) can be identified. Thus, to study the effect of agents on reducing cholesterol

transport from the gut to the blood or lymph, or for reducing LDL or serum cholesterol levels, for example, in a clinical trial, cells can be isolated and RNA prepared and analyzed for the levels of expression of ABC1 and other genes implicated in the disorder. The levels of gene expression can be quantified by Northern blot analysis or RT-PCR, or, alternatively, by measuring the amount of protein produced, by one of a number of methods known in the art, or by measuring the levels of biological activity of ABC1 or other genes. In this way, the gene expression can serve as a marker, indicative of the physiological response of the cells to the agent. Accordingly, this response state may be determined before, and at various points during, treatment of the individual with the agent.

In a preferred embodiment, the present invention provides a method for monitoring the effectiveness of treatment of a subject with an agent (e.g., an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate identified by the screening assays described herein) including the steps of (i) obtaining a pre-administration sample from a subject prior to administration of the agent; (ii) detecting the level of expression of an ABC1 protein, mRNA, or genomic DNA in the preadministration sample; (iii) obtaining one or more post-administration samples from the subject; (iv) detecting the level of expression or activity of the ABC1 protein, mRNA, or genomic DNA in the post-administration samples; (v) comparing the level of expression or activity of the ABC1 protein, mRNA, or genomic DNA in the pre-administration sample with the ABC1 protein, mRNA, or genomic DNA in the post administration sample or samples; and (vi) altering the administration of the agent to the subject accordingly. For example, administration of the agent may be desirable to decrease the expression or activity of ABC1.

The *ABC1* gene or a fragment thereof can be used as a tool to express the protein in an appropriate cell *in vitro* or *in vivo* (gene therapy), or can be cloned into expression vectors which can be used to produce large enough amounts of ABC1 protein to use in *in vitro* assays for drug screening. Expression systems which may be employed include baculovirus, herpes virus, adenovirus, adeno-associated virus, bacterial systems, and eucaryotic systems such as CHO cells. Naked DNA and DNA-liposome complexes can also be used.

Assays of ABC1 activity includes binding to intracellular interacting proteins; interaction with a protein that modulates ABC1 activity; interaction with HDL particles or constituents; interaction with other proteins which facilitate interaction with HDL or its constituents; and measurement of cholesterol efflux. Furthermore, assays may be based upon the molecular dynamics of macromolecules, metabolites and ions by means of

fluorescent-protein biosensors.

Alternatively, the effect of candidate modulators on expression or activity may be measured at the level of ABC1 protein production using the same general approach in combination with standard immunological detection techniques, such as Western blotting or immunoprecipitation with an ABC1-specific antibody. Again, useful modulators are identified as those which produce a change in ABC1 polypeptide production. Agonists may also affect ABC1 activity without any effect on expression level.

Agonists, antagonists, or mimetics found to be effective at modulating the level of cellular ABC1 expression or activity may be confirmed as useful in animal models (for example, mice, pigs, rabbits, or chickens).

A compound that promotes a decrease in ABC1 expression or activity is considered particularly useful in the invention; such a molecule may be used, for example, as a therapeutic to decrease the level or activity of native, cellular ABC1 and thereby reduce cholesterol transport from the gut to the blood or lymph, or reduce LDL or serum cholesterol levels in an animal (for example, a human).

One method for decreasing ABC biological activity is to decrease the stabilization of the ABC protein or to prevent its degradation.

In one example, cells expressing an ABC1 polypeptide having a mutation are transiently metabolically labeled during translation and the half-life of the ABC1 polypeptide is determined using standard techniques. Mutations that decrease the half-life of an ABC1polypeptide are ones that decrease ABC1 protein stability.

The Cholesterol Efflux Assay as a Drug Screen

A cholesterol efflux assay measures the ability of cells to transfer cholesterol to an extracellular acceptor molecule and is dependent on ABC1 function. A standard cholesterol efflux assay is set out in Marcil et al., *Arterioscler. Thromb. Vasc. Biol.* 19:159-169, 1999, incorporated by reference herein for all purposes. Prior to this invention, this assay has not been used to identify compounds useful for reducing cholesterol transport from the gut to the blood or lymph, or for reducing LDL or serum cholesterol levels.

In this procedure, cells are loaded with radiolabeled cholesterol by any of several biochemical pathways. Cholesterol efflux of cells is measured after incubation for various times (typically 0 to 24 hours) in the presence of HDL3 or purified ApoAI. Cholesterol efflux is determined as the percentage of total cholesterol in the culture medium after various times of incubation. Increased ABC1 expression levels and/or biological activity are associated with increased efflux while decreased levels of ABC1 expression and/or biological activity are associated with decreased cholesterol efflux.

This assay can be readily adapted to the format used for drug screening, which may consist of a multi-well (e.g., 96-well) format. Modification of the assay to optimize it for drug screening would include scaling down and streamlining the procedure, modifying the labeling method, using a different cholesterol acceptor, altering the incubation time, and changing the method of calculating cholesterol efflux. In all these cases, the cholesterol efflux assay remains conceptually the same, though experimental modifications may be made.

For high throughput, fluorescent lipids can be used to measure ABC1-catalyzed lipid efflux. For phospholipids, a fluorescent precursor, C6-NBD-phosphatidic acid, can be used. This lipid is taken up by cells and dephosphorylated by phosphatidic acid phosphohydrolase. The product, NBD-diglyceride, is then a precursor for synthesis of glycerophospholipids like phosphatidylcholine. The efflux of NBD-phosphatidylcholine can be monitored by detecting fluorescence resonance energy transfer (FRET) of the NBD to a suitable acceptor in the cell culture medium. This acceptor can be rhodamine-labeled phosphatidylethanolamine, a phospholipid that is not readily taken up by cells. The use of short-chain precursors obviates the requirement for the phospholipid transfer protein in the media. For cholesterol, NBD-cholesterol ester can be reconstituted into LDL. The LDL can efficiently deliver this lipid to cells via the LDL receptor pathway. The NBD-cholesterol esters are hydrolyzed in the lysosomes, resulting in NBD-cholesterol that can now be transported back to the plasma membrane and efflux from the cell. The efflux can be monitored by the aforementioned FRET assay in which NBD transfers its fluorescence resonance energy to the rhodamine-phosphatidylethanoline acceptor.

Protein-based assays

ABC1 polypeptide (purified or unpurified) can be used in an assay to determine its ability to bind another protein (including, but not limited to, proteins found to specifically interact with ABC1). The effect of a compound on that binding is then determined. Useful ABC1 proteins include wild-type and mutant ABC1 proteins or protein fragments, in a recombinant form or endogenously expressed.

Protein Interaction Assays

ABC1 protein (or a polypeptide fragment thereof or an epitope-tagged form or fragment thereof) is harvested from a suitable source (e.g., from a prokaryotic expression system, eukaryotic cells, a cell-free system, or by immunoprecipitation from ABC1-expressing cells). The ABC1 polypeptide is then bound to a suitable support (e.g., nitrocellulose or an antibody or a metal agarose column in the case of, for example, a his-tagged form of ABC1). Binding to the support is preferably done under conditions that

allow proteins associated with ABC1 polypeptide to remain associated with it. Such conditions may include use of buffers that minimize interference with protein-protein interactions. The binding step can be done in the presence and absence of compounds being tested for their ability to interfere with interactions between ABC1 and other molecules. If desired, other proteins (e.g., a cell lysate) are added, and allowed time to associate with the ABC polypeptide. The immobilized ABC1 polypeptide is then washed to remove proteins or other cell constituents that may be non-specifically associated with it the polypeptide or the support. The immobilized ABC1 polypeptide is then dissociated from its support, and so that proteins bound to it are released (for example, by heating), or, alternatively, associated proteins are released from ABC1 without releasing the ABC1 polypeptide from the support. The released proteins and other cell constituents can be analyzed, for example, by SDS-PAGE gel electrophoresis, Western blotting and detection with specific antibodies, phosphoamino acid analysis, protease digestion, protein sequencing, or isoelectric focusing. Normal and mutant forms of ABC1 can be employed in these assays to gain additional information about which part of ABC1 a given factor is binding to. In addition, when incompletely purified polypeptide is employed, comparison of the normal and mutant forms of the protein can be used to help distinguish true binding proteins.

The foregoing assay can be performed using a purified or semipurified protein or other molecule that is known to interact with ABC1. This assay may include the following steps.

1. Harvest ABC1 protein and couple a suitable fluorescent label to it;

2. Label an interacting protein (or other molecule) with a second, different fluorescent label. Use dyes that will produce different quenching patterns when they are in close proximity to each other vs. when they are physically separate (i.e., dyes that quench each other when they are close together but fluoresce when they are not in close proximity);

3. Expose the interacting molecule to the immobilized ABC1 in the presence or absence of a compound being tested for its ability to interfere with an interaction between the two; and

4. Collect fluorescent readout data.

Another possible assay is the Fluorescent Resonance Energy Transfer (FRET) assay. This assay can be performed as follows.

1. Provide ABC1 protein or a suitable polypeptide fragment thereof and couple a suitable FRET donor (e.g.,. nitro-benzoxadiazole (NBD)) to it;

2. Label an interacting protein (or other molecule) with a FRET acceptor (e.g.,

rhodamine);

3. Expose the acceptor-labeled interacting molecule to the donor-labeled ABC1 in the presence or absence of a compound being tested for its ability to interfere with an interaction between the two; and

4. Measure fluorescence resonance energy transfer.

Quenching and FRET assays are related. Either one can be applied in a given case, depending on which pair of fluorophores is used in the assay.

Membrane permeability assay

The ABC1 protein can also be tested for its effects on membrane permeability. For example, beyond its putative ability to translocate lipids, ABC1 might affect the permeability of membranes to ions. Other related membrane proteins, most notably the cystic fibrosis transmembrane conductance regulator and the sulfonylurea receptor, are associated with and regulate ion channels.

ABC1 or a fragment of ABC1 is incorporated into a synthetic vesicle, or, alternatively, is expressed in a cell and vesicles or other cell sub-structures containing ABC1 are isolated. The ABC1-containing vesicles or cells are loaded with a reporter molecule (such as a fluorescent ion indicator whose fluorescent properties change when it binds a particular ion) that can detect ions (to observe outward movement), or alternatively, the external medium is loaded with such a molecule (to observe inward movement). A molecule which exhibits differential properties when it is inside the vesicle compared to when it is outside the vesicle is preferred. For example, a molecule that has quenching properties when it is at high concentration but not when it is at another low concentration would be suitable. The movement of the charged molecule (either its ability to move or the kinetics of its movement) in the presence or absence of a compound being tested for its ability to affect this process can be determined.

In another assay, membrane permeability is determined electro-physiologically by measuring ionic influx or efflux mediated by or modulated by ABC1 by standard electrophysiological techniques. A suitable control (e.g., TD cells or a cell line with very low endogenous ABC1 expression) can be used as a control in the assay to determine if the effect observed is specific to cells expressing ABC1.

In still another assay, uptake of radioactive isotopes into or out of a vesicle can be measured. The vesicles are separated from the extravesicular medium and the radioactivity in the vesicles and in the medium is quantitated and compared.

Nucleic acid-based assays

ABC1 nucleic acid may be used in an assay based on the binding of factors necessary for ABC1 gene transcription. The association between the *ABC1* DNA and the binding factor may be assessed by means of any system that discriminates between protein-bound and non-protein-bound DNA (e.g., a gel retardation assay). The effect of a compound on the binding of a factor to *ABC1* DNA is assessed by means of such an assay. In addition to *in vitro* binding assays, *in vivo* assays in which the regulatory regions of the ABC1 gene are linked to reporter genes can also be performed.

Assays measuring stability of ABC1 protein or mRNA

A cell-based or cell-free system can be used to screen for compounds based on their effect on the half-life of *ABC1* mRNA or ABC1 protein. The assay may employ labeled mRNA or protein. Alternatively, *ABC1* mRNA may be detected by means of specifically hybridizing probes or a quantitative PCR assay. Protein can be quantitated, for example, by fluorescent antibody-based methods.

In vitro mRNA stability assay

1. Isolate or produce, by *in vitro* transcription, a suitable quantity of ABC1 mRNA;

2. Label the ABC1 mRNA;

3. Expose aliquots of the mRNA to a cell lysate in the presence or absence of a compound being tested for its ability to modulate ABC1 mRNA stability;

4. Assess intactness of the remaining mRNA at suitable time points.

In vitro protein stability assay

1. Express a suitable amount of ABC1 protein;

2. Label the protein;

3. Expose aliquots of the labeled protein to a cell lysate in the presence or absence of a compound being tested for its ability to modulate ABC1 protein stability;

4. Assess intactness of the remaining protein at suitable time points

In vivo mRNA or protein stability assay

1. Incubate cells expressing ABC1 mRNA or protein with a tracer (radiolabeled ribonucleotide or radiolabeled amino acid, respectively) for a very brief time period (e.g., five minutes) in the presence or absence of a compound being tested for its effect on mRNA or protein stability;

2. Incubate with unlabeled ribonucleotide or amino acid; and

3. Quantitate the ABC1 mRNA or protein radioactivity at time intervals beginning with the start of step 2 and extending to the time when the radioactivity in ABC1 mRNA or protein has declined by approximately 80%. It is preferable to separate the intact or mostly

intact mRNA or protein from its radioactive breakdown products by a means such as gel electrophoresis in order to quantitate the mRNA or protein.

Assays measuring inhibition of dominant negative activity

Mutant ABC1 polypeptides are likely to have dominant negative activity (i.e., activity that interferes with wild-type ABC1 function). An assay for a compound that can interfere with such a mutant may be based on any method of quantitating normal ABC1 activity in the presence of the mutant. For example, normal ABC1 facilitates cholesterol efflux, and a dominant negative mutant would interfere with this effect. The ability of a compound to counteract the effect of a dominant negative mutant may be based on cellular cholesterol efflux, or on any other normal activity of the wild-type ABC1 that was inhibitable by the mutant.

Assays measuring phosphorylation

The effect of a compound on ABC1 phosphorylation can be assayed by methods that quantitate phosphates on proteins or that assess the phosphorylation state of a specific residue of a ABC1. Such methods include but are not limited to ^{32}P labelling and immunoprecipitation, detection with antiphosphoamino acid antibodies (e.g., antiphosphoserine antibodies), phosphoamino acid analysis on 2-dimensional TLC plates, and protease digestion fingerprinting of proteins followed by detection of ^{32}P-labeled fragments.

Assays measuring other post-translational modifications

The effect of a compound on the post-translational modification of ABC1 is based on any method capable of quantitating that particular modification. For example, effects of compounds on glycosylation may be assayed by treating ABC1 with glycosylase and quantitating the amount and nature of carbohydrate released.

Assays measuring ATP binding

The ability of ABC1 to bind ATP provides another assay to screen for compounds that affect ABC1. ATP binding can be quantitated as follows.

1. Provide ABC1 protein at an appropriate level of purity and reconsititute it in a lipid vesicle;

2. Expose the vesicle to a labeled but non-hydrolyzable ATP analog (such as gamma ^{35}S-ATP) in the presence or absence of compounds being tested for their effect on ATP binding. Note that azido-ATP analogs can be used to allow covalent attachment of the azido-ATP to protein (by means of U.V. light), and permit easier quantitation of the amount of ATP bound to the protein.

3. Quantitate the amount of ATP analog associated with ABC1

Assays measuring ATPase activity

Quantitation of the ATPase activity of ABC1 can also be assayed for the effect of compounds on ABC1. This is preferably performed in a cell-free assay so as to separate ABC1 from the many other ATPases in the cell. An ATPase assay may be performed in the presence or absence of membranes, and with or without integration of ABC1 protein into a membrane. If performed in a vesicle-based assay, the ATP hydrolysis products produced or the ATP hydrolyzed may be measured within or outside of the vesicles, or both. Such an assay may be based on disappearance of ATP or appearance of ATP hydrolysis products. For high-throughput screening, a coupled ATPase assay is preferable. For example, a reaction mixture containing pyruvate kinase and lactate dehydrogenase can be used. The mixture includes phosphoenolpyruvate (PEP), nicotinamide adenine dinucleotide (NAD+), and ATP. The ATPase activity of ABC1 generates ADP from ATP. The ADP is then converted back to ATP as part of the pyruvate kinase reaction. The product, pyruvate, is then converted to lactate. The latter reaction generates a colored quinone (NADH) from a colorless substrate (NAD+), and the entire reaction can be monitored by detection of the color change upon formation of NADH. Since ADP is limiting for the pyruvate kinase reaction, this coupled system precisely monitors the ATPase activity of ABC1.

Animal Model Systems

Compounds identified as having activity in any of the above-described assays are subsequently screened in any available animal model system, including, but not limited to, pigs, rabbits, and WHAM chickens. Test compounds are administered to these animals according to standard methods. Test compounds may also be tested in mice bearing mutations in the ABC1 gene. Additionally, compounds may be screened for their ability to enhance an interaction between ABC1 and any HDL particle constituent such as ApoAI, ApoAII, or ApoE.

Knock-out mouse model

An animal, such as a mouse, that has had one or both ABC1 alleles inactivated (e.g., by homologous recombination) is a preferred animal model for screening for compounds that reduce exogenous cholesterol transport from the gut lumen to the blood or lymph and for the regulation and treatment of cardiovascular disorders (such as high LDL or serum cholesterol levels), obesity, elevated body-weight index and other disorders relating to lipid metabolism. Such an animal can be produced using standard techniques. In addition to the initial screening of test compounds, the animals having mutant ABC1 genes are useful for further testing of efficacy and safety of drugs or agents first identified using one of the other screening methods described herein. Cells taken from the animal and placed in culture can

also be exposed to test compounds.

WHAM chickens: an animal model for low HDL cholesterol

Wisconsin Hypo-Alpha Mutant (WHAM) chickens arose by spontaneous mutation in a closed flock. Mutant chickens came to attention through their a Z-linked white shank and white beak phenotype referred to as 'recessive white skin' (McGibbon, 1981) and were subsequently found to have a profound deficiency of HDL (Poernama et al., 1990).

This chicken low HDL locus (Y) is Z-linked, or sex-linked. (In birds, females are ZW and males are ZZ). Genetic mapping placed the Y locus on the long arm of the Z chromosome (Bitgood, 1985), proximal to the ID locus (Bitgood, 1988). Examination of current public mapping data for the chicken genome mapping project, ChickMap (maintained by the Roslin Institute; http://www.ri.bbsrc.ac.uk/chickmap/ChickMapHomePage.html) showed that a region of synteny with human chromosome 9 lies on the long arm of the chicken Z chromosome (Zq) proximal to the ID locus. Evidence for this region of synteny is the location of the chicken aldolase B locus (ALDOB) within this region. The human ALDOB locus maps to chromosome 9q22.3 (The Genome Database, http://gdbwww.gdb.org/), not far from the location of human ABC1. This comparison of maps showed that the chicken Zq region near chicken ALDOB and the human 9q region near human ALDOB represent a region of synteny between human and chicken.

We predicted that ABC1 is mutated in WHAM chickens. In support of this, we have identified an E to K mutation at a position that corresponds to amino acid 89 of human ABC1. This non-conservative substitution is at a position that is conserved among human, mouse, and chicken, indicating that it is in a region of the protein likely to be of functional importance.

Discovery of the WHAM mutation in the amino-terminal portion of the ABC1 protein also establishes the importance of the amino-terminal region. This region may be critical because of association with other proteins required to carry out cholesterol efflux or related tasks. It may be an important regulatory region (there is a phosphorylation site for casein kinase near the mutated residue), or it may help to dictate a precise topological relationship with cellular membranes (the N-terminal 60 amino acid region contains a putative membrane-spanning or membrane-associated segment).

The amino-terminal region of the protein (up to the first 6-TM region at approximately amino acid 639) is an ideal tool for screening factors that affect ABC1 activity. It can be expressed as a truncated protein in ABC1 wild type cells in order to test for interference of the normal ABC1 function by the truncated protein. If the fragment acts

in a dominant negative way, it could be used in immunoprecipitations to identify proteins that it may be competing away from the normal endogenous protein.

The C-terminus also lends itself to such experiments, as do the intracellular portions of the molecule, expressed as fragments or tagged or fusion proteins, in the absence of transmembrane regions.

Since it is possible that there are several genes in the human genome which affect cholesterol efflux, it is important to establish that any animal model to be used for a human genetic disease represents the homologous locus in that animal, and not a different locus with a similar function. The evidence above establishes that the chicken Y locus and the human chromosome 9 low HDL locus are homologous. WHAM chickens are therefore an important animal model for the identification of drugs that modulate cholesterol efflux, and as such are useful for reducing cholesterol transport from the gut lumen to the blood or lymph and for the regulation and treatment of cardiovascular disorders (such as high LDL or serum cholesterol levels), obesity, elevated body-weight index and other disorders relating to lipid metabolism.

Compounds of the Invention

In general, novel compounds and therapeutic agents for reducing cholesterol transport from the gut lumen to the blood or lymph and for the regulation and treatment of cardiovascular disorders (such as high LDL or serum cholesterol levels), obesity, elevated body-weight index and other disorders relating to lipid metabolism are identified from large libraries of both natural product or synthetic (or semi-synthetic) extracts or chemical libraries according to methods known in the art. Those skilled in the field or drug discovery and development will understand that the precise source of test extracts or compounds is not critical to the screening procedure(s) of the invention. Accordingly, virtually any number of chemical extracts or compounds can be screened using the exemplary methods described herein. Examples of such extracts or compounds include, but are not limited to, plant-, fungal-, prokaryotic- or animal-based extracts, fermentation broths, and synthetic compounds, as well as modification of existing compounds. Numerous methods are also available for generating random or directed synthesis (e.g., semi-synthesis or total synthesis) of any number of chemical compounds, including, but not limited to, saccharide-, lipid-, peptide-, and nucleic acid-based compounds. Synthetic compound libraries are commercially available from Brandon Associates (Merrimack, NH) and Aldrich Chemical (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are commercially available from a number of sources, including Biotics (Sussex, UK), Xenova (Slough, UK), Harbor Branch Oceangraphics

Institute (Ft. Pierce, FL), and PharmaMar, U.S.A. (Cambridge, MA). In addition, natural and synthetically produced libraries are produced, if desired, according to methods known in the art, e.g., by standard extraction and fractionation methods. Furthermore, if desired, any library or compound is readily modified using standard chemical, physical, or biochemical methods.

Typically, a screening assay, such as a high throughput screening assay, will identify several or even many compounds which modulate the activity of the assay protein. The compound identified by the screening assay may be further modified before it is used in humans as the therapeutic agent. Typically, combinatorial chemistry is performed on the modulator, to identify possible variants that have improved absorption, biodistribution, metabolism and/or excretion, or other important therapeutic aspects. The essential invariant is that the improved compounds share a particular active group or groups which are necessary for the desired modulation of the target protein. Many combinatorial chemistry techniques are well known in the art. Each one adds or deletes one or more constituent moieties of the compound to generate a modified analog, which analog is again assayed to identify compounds of the invention. Thus, as used in this invention, therapeutic compounds identified using an ABC1 screening assay of the invention include actual compounds so identified, and any analogs or combinatorial modifications made to a compound which is so identified which are useful for treatment of the disorders claimed herein.

In addition, those skilled in the art of drug discovery and development readily understand that methods for dereplication (e.g., taxonomic dereplication, biological dereplication, and chemical dereplication, or any combination thereof) or the elimination of replicates or repeats of materials already known for their abilities in reducing cholesterol transport from the gut lumen to the blood or lymph and for the regulation and treatment of cardiovascular disorders (such as high LDL or serum cholesterol levels), obesity, elevated body-weight index and other disorders relating to lipid metabolism should be employed whenever possible.

When a crude extract is found to be capable of reducing cholesterol transport from the gut lumen to the blood or lymph and for the regulation and treatment of cardiovascular disorders (such as high LDL or serum cholesterol levels), obesity, elevated body-weight index and other disorders relating to lipid metabolism, further fractionation of the positive lead extract is necessary to isolate chemical constituent responsible for the observed effect. Thus, the goal of the extraction, fractionation, and purification process is the careful characterization and identification of a chemical entity within the crude extract having these

desired activities. The same in vivo and in vitro assays described herein for the detection of activities in mixtures of compounds can be used to purify the active component and to test derivatives thereof. Methods of fractionation and purification of such heterogeneous extracts are known in the art. If desired, compounds shown to be useful agents for the treatment of pathogenicity are chemically modified according to methods known in the art. Compounds identified as being of therapeutic value are subsequently analyzed using any standard animal model for the desired disease or condition known in the art.

It is understood that compounds that indirectly modulate ABC1 activity, for example by modulation of proteins that modulate or are modulated by ABC1, may also be useful compounds for the desired purposes of the invention. Such compositions which are identified using the screening assays of this invention are also claimed.

Because one of the objects of the invention is to inhibit cholesterol transport in the gut but to not inhibit the assembly of HDL particles in peripheral tissues, certain features of preferred compositions of the invention can be identified. In particular, compositions which act locally in the gut or intestinal wall, but which do not circulate widely in the body are preferred. This object may be achieved with compounds which either are incapable of being transported by the blood or lymph or other extra-cellular fluid or particle. This object may also be achieved by obtaining compounds with limited in vivo stability (i.e. short half life upon oral administration) or which are subject to rapid metabolism to inert analogs after absorption by the intestinal wall.

As described previously, analogs of sulfonylureas are likely candidate compounds. However, sulfonylureas that are routinely used by diabetics are not useful in the invention to the extent that they cause the undesireable side-effect - in non-diabetic patients - of increased insulin secretion by the pancreas. Therefore, preferred compounds of the invention are inhibitors of ABC1 that either do not disperse significantly beyond the gut; do not cause unacceptable inhibition of ABC1 in peripheral tissues; and do not cause unacceptable side-effects.

Therapy using compositions of the invention

Compositions of the invention, including but not limited to compounds that modulate biological activity or expression of ABC1 identified using any of the methods disclosed herein, or any preferred analogs of such compositions, may be administered with a pharmaceutically-acceptable diluent, carrier, or excipient, in unit dosage form. Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer such compositions to patients. Although oral administration is

preferred, any appropriate route of administration may be employed, for example, intravenous, perenteral, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, or aerosol administration. Therapeutic formulations may be in the form of liquid solutions or suspension; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are found in, for example, Remington: *The Science and Practice of Pharmacy*, (19th ed.) ed. A.R. Gennaro AR., 1995, Mack Publishing Company, Easton, PA. Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated napthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems for agonists of the invention include ethylenevinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, or example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

A preferred embodiment for use of the compositions of the invention is for combination therapy employing a therapeutic agent of the invention which modulates or inhibits ABC1 activity in the gut in combination, simultaneous or sequential, with another agent which inhibits endogenous cholesterol synthesis, such as but not limited to a "statin" or HMGCoA reductase inhibitor, etc. This combination therapy is preferred in instances where inhibition of both exogenous cholesterol uptake from the gut and inhibition of endogenous cholesterol synthesis are desired. Therapeutic agents employed in this combination therapy are preferably oral compounds. In a preferred embodiment, the ABC1 inhibitor does not disperse beyond the lamina propria of the gut (i.e. it stays largely in the gut), whereas the inhibitor of endogenous cholesterol synthesis circulates to sites of endogenous cholesterol synthesis in the body.

EXAMPLES

WHAM chickens.

The Wisconsin Mutant Hypoalpha (WHAM) chickens were discovered in 1981 in a flock of chickens maintained by the University of Wisconsin. The WHAM chickens have

white skin and white beaks and have colorless rather than yellow serum, all due to the absence of carotenoids. The trait is inherited as a recessive sex-linked mutation on the Z-chromosome. These animals also have a severe deficiency of high density lipoprotein (HDL). Metabolic studies led to some degree of understanding of the defect in HDL metabolism. When ^{125}I-labeled HDL particles were injected into WHAM chickens, their disappearance from the circulation was only moderately increase relative to normal chickens. However, when lipid-free ^{125}I-apo-A1 was injected, it was removed from the circulation four-fold more rapidly in WHAM chickens compared to normal chickens, by the kidneys. Because apo-A1 synthesis and secretion is normal in the WHAM chickens, another factor had to affect the stability of apo-A1. Analysis of the serum phospholipids showed a 70% reduction, implying that the primary defect is in phospholipid efflux and demonstrated than an extracellular event is required for the formation of stable HDL particles.

The lipoprotein profiles of WHAM chicken and Tangier patient plasma show a similarly pronounced loss of HDL. IN addition, both plasma were found to show a decrease in plasma phospholipid levels. Two-dimensional thin-layer chromatography showed that the most pronounced phospholipid deficiency was in phosphotidylcholine and sphingomyelin.

A genetic study of the WHAM chicken genetic revealed that the location to which the mutant gene mapped adjacent to genes which in turn are adjacent to ABC1 on the human genome on chromosome 9. Shown in Fig. 1 is a map comparing the synteny between the WHAM mutation and the human ABC1 gene. Markers mapped genetically or physically are indicated by dashed arrows. Genes mapped only cytogenetically are positioned relative to other markers with the cytogenetic location in brackets.

To investigate the gene comparison, the coding region of the ABC1 genes from humans and the WHAM chickens were compared. The human and chicken genes are 78% identical at the nucleotide level and 85% identical (with 92% homology) at the amino acid level. The sequence of the normal and the WHAM chickens were identical with the exception of a G to an A transition in the WHAM DNA at nucleotide 265, corresponding to a glutamic acid to lysine substitution at amino acid position 89. Studies of the DNA of WHAM chickens, conducted by RFLP analysis, revealed that the mutation segregates with the phenotype of HDL deficiency.

Referring specifically again to Fig. 1, the WHAM mutation maps to a Z chromosome region syntenic to the 9q31.1 location of human ABC1. To the left is the chicken Z chromosome combined genetic and cytogenetic map. To the right is a combined

human genetic and cytogenetic map. Positions of markers mapped genetically or physically are indicated by dashed arrows. Genes mapped only cytogenetically are positioned relative to other markers with the cytogenetic location in brackets. WHAM was genetically mapped relative to ID and B [the relative distances and the calculated WHAM-B distance are indicated, (1,2).]

At (B) in Fig. 1, the illustration conveys that the WHAM chicken ABC1 gene has a single amino acid substitution (E89K) relative to normal White Leghorn chicken. Total liver RNA from WHAM and normal male chickens was subjected to standard RT-PCR and sequencing methods (left panel) using primers corresponding to the cDNA sequences most conserved between human and mouse ABC1. The open reading frame (corresponding to amino acids 27 to 2261) was sequenced, revealed a single homozygous G to A transition in WHAM cDNA at position 265. (Numbering of nucleotides and amino acids is according to the new, longer open reading frame of human ABC1). The same alteration was observed in PCR product of chicken genomic DNA (right panel).

As also shown in Fig. 1, RFLP analysis confirmed the presence of the WHAM mutation in genomic DNA. Genomic DNA from normal and mutant homozygous male and hemizygous female chickens was amplified using PCR primers

forward:

5'-GTCACTTCCCAAACAAAGCTA-3' SEQ ID No.

reverse:

5'-ATGGACGCATTGAAGTTTCC-3' SEQ ID No.

flanking the WHAM mutation, then the PCR products digested with HinfI. The WHAM alteration destroys a HinfI site, resulting in a 142 bp uncut fragment rather than the 106 bp and 36 bp fragments of normal chickens. The chicken sex chromosomes of each bird tested are indicated below the photo; male chickens are ZZ, female chickens are ZW.

The glutamate residue at the position of the non conservative E89K substitution is conserved between human (CAA10005), mouse (CAA53530), Takifugu rubripes ('fugu'), and chicken. The WHAM mutation is thus predicted to have a deleterious effect on activity of the ABC1 protein. The fugu amino acid sequence was predicted from nucleotide sequence of a cosmid containing the fugu ABC1 gene. Bitgood JJ. 1985, "Additional linkage relationships within the Z chromosome of the chicken," *Poultry Science* 64: 2234-8 Bitgood JJ. 1988, "Linear relationship of the loci for barring, dermal melanin inhibitor, and recessive white skin on the chicken Z chromosome," *Poultry Sci.* 67: 530-3.

Dietary cholesterol and WHAM chickens

Fig. 2 illustrates the results of time courses of plasma cholesterol in control and WHAM chickens on a cholesterol-free or high-cholesterol diet. The basal diet (ad libitum) was acorn-soy- based diet to which 12.4% (by weight) lard was added. By calculation,the diet contained 14% fat by weight or 37% as total calories. The two dietary treatments consisted of the basal (cholesterol-free) diet and the basal plus 4% cholesterol diet. The diets were each fed to two groups of chickes, each group comprising 10 animals, for 28 weeks.

This example demonstrates the effect of inhibition of ABC1 (here demonstrated by an inactivating mutation in the gene, but also obtainable by inhibitors of ABC1 identified by the screening assays of the invention) on cholesterol absorption by the WHAM chicken. Cholesterol transport from the lumen of the gut to the blood or lymph is blocked or eliminated by inhibition of the ABC1 gene. In this case the genetic mutation is a surrogate for an antagonist of the ABC1 protein.

Cholesterol retention

The WHAM chickens, like Tangier patients, show evidence of cholesterol ester retention. Like Tangier patients, the WHAM chickens have large non-osmiophilic drops in the cytoplasm of splenic macrophages. In addition, electro-micrographs of the intestinal wall of the chicken (control and WHAM), show very specific accumulation of cholesterol in non-endothelial cells of the lamina propria. See Figure 3 which shows those cells. In Figure 3, cells of the intestinal wall, which are not columnar epithelium cells, from the WHAM chickens contain giant lipid droplets. In Fig. 3, photograph (A) shows normal intestine showing the microvilli at the apical surface. Spaces represent normal sites where chylomicron particles are secreted. Image (B) shows higher magnification of area shown in lower rectangle in (A). Image (C) is a higher magnification of intestinal wall from upper rectangle in (A). Photograph (D) shows the WHAM intestine showing apical surface, the absence of spaces between the cells, and accumulation of vesicles. Image (E) is a higher magnification of area in rectangle in (A). Note the abundance of vesicles relative to the control section in (B). Image (F) is a higher magnification of intestinal wall area just above the top of (D). Lipid inclusions 1.5-2.0 μm in diameter. Bar = 2.5 μm in (A) and (D) and 0.6 μm in all other panels.

We conclude from this evidence that the WHAM chickens are able to absorb cholesterol from the intestinal lumen but are unable to transport that cholesterol out of the epithelial cells into the blood stream. This explains the accumulation of cholesterol in those cells.

Sulfonylurea compounds

A drawback in the use of some sulfonylurea compounds is that such compounds can have unwanted activity in stimulating insulin secretion. Therefore, it is contemplated that a screening program be conducted to identify and assess either sulfonylurea or other compounds which have activity in inhibiting ABC1 but which do not stimulate unwanted insulin production. It is envisioned that this screen can be done by giving the compound orally to test animals. A tracer of radioactively labeled cholesterol can then be given to the animals. At various time points after administration (1, 2, 3, 4, 6, 8, 12, 16, 24, 36, and 48 hours), blood samples would be taken and the amount of the isotope in cholesterol and cholesterol ester of chylomicron particles would be sampled. The chylomicron fraction would be obtained by centrifuging the plasma in an ultracentrifuge (20,000 rpm in a 40.3 rotor for 20 minutes). The chylomicrons float to the top and are removed by aspiration. The area under the isotope amount versus time curve would then indicate the amount of tracer that has entered into the bloodstream. When divided by the amount initially administered in an oral dose, the percent of cholesterol that traveled from the intestinal lumen all the way into the bloodstream can be computed. Inhibitors of the function of the ABC1 protein or gene activity will reduce this amount. In *in vivo* assays, effects on insulin secretion can be measured by standard blood assays known in the art, such as a quantitative insulin radio-immunoassay.

In vitro screening assays for identifying unwanted activity in stimulating insulin secretion are also standard and known in the art. In a typical assay, islet cells are isolated from the pancreas by a collagenase digest. Cells are cultured; then exposed to the candidate substance. Insulin secretion by the cultured cells is measured by a quantitative radio-immunoassay. Candidate compounds which increase the level of insulin secretion are rejected as having undesirable side effects for the desired uses of the invention herein.

Inhibiting ABC1 activity

ABC1 activity can be inhibited genetically or chemically. It is known that sulfonylurea drugs inhibit ABC1 activity. Shown in Fig. 4 is the results of a study demonstrating that effect. Mouse macrophages (J774) were labeled with ^{3}H-cholesterol (2 μCi/ml) for 24 hours in 1% v/v fetal bovine serum. Following the labeling, the cells were equilibrated with 0.2% de-fatted bovine serum albumin in RPMI growth medium. Cholesterol efflux was initiated with the addition of 20μg/ml of human apolipoprotein-A1 in the presence of the indicated concentrations of Glyburide, a sulfonylurea compound. After 24 hours, the medium was collected, centrifuged, and an aliquot collected for radioactivity determination by liquid scintillation counting.

This study demonstrates that the efficacy of a compound in inhibiting ABC1 activity

can be measured in a cellular assay. This same assay can be used to test other compounds for ABC1 inhibitory activity.

The preceding examples and specification are illustrations of the invention which are non-limiting examples of the invention more generally described by the claims below.